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Effect of historical predation pressure and current predation risk on genetically determined behaviour of the nine-spined stickleback

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Tiivistelmä – Referat – Abstract

Behaviour is a key component in ecological interactions and studying its role in adaptation is central in our understanding of natural selection and phenotypic variation in the wild. Predation is an important driver shaping animal behaviour in the wild, since predators have been shown to select against particular behavioural phenotypes. However, it is not easy to demonstrate that specific behaviours are adaptive to certain levels of predation, since behaviours are often correlated with each other forming multivariate phenotypes and display notable phenotypic plasticity.

I studied how predation shapes genetically determined behaviour of the nine-spined stickleback (*Pungitius pungitius*) through variation in historical predation pressures and by inducing phenotypic plasticity. This was achieved through rearing 65 full-sib families of nine-spined sticklebacks derived from four coastal marine (predator-sympatric) and four pond (predator-naïve) populations in a common garden experiment and quantifying their behaviour in the laboratory in the presence and absence of natural predators. Since the fish used were F1-generation offspring from artificial crosses, I was also able to estimate the heritability and genetic correlations of the studied behaviours.

Pond sticklebacks tended to be more explorative and took more risks during foraging than marine sticklebacks regardless of predation risk. In all fish, predator presence decreased the propensity to take risks during foraging, but not exploration tendency. Since the fish were reared in a common garden setting, there is a genetic basis for these population differences. Both behaviours were heritable in all populations.

In this study, I observed genetically based and heritable behavioural differences between pond and marine stickleback populations. Despite showing similar levels of behavioural plasticity as marine sticklebacks, pond sticklebacks were still inappropriately active in the presence of predators and would have a low survival probability in a predator-sympatric environment. In risk-taking during foraging, the behavioural trend caused by acute predation risk was directionally the same as that caused by evolutionary history of predation risk, implying that the behavioural differentiation between marine and pond populations in this behaviour is due to predation. These results provide evidence of local adaptation in behaviour to differing levels of predation in these populations, and that this adaptation comes about as differences in the overall level of behaviour rather than in phenotypic plasticity.

Avainsanat – Nyckelord – Keywords
Behavioural evolution, local adaptation, behavioural plasticity, behavioural syndromes, quantitative genetic variance, *Pungitius*, stickleback

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Tiivistelmä – Referat – Abstract

Käyttäytyminen on oleellinen tekijä ekologisissa vuorovaikutussuhteissa, ja luonnonvalinnan ja fenotyyppisen monimuotoisuuden ymmärtämisen kannalta onkin keskeistä tutkia sen roolia sopeutumisessa. Saalistus on tärkeä eläinten käyttäytymistä muokkaava tekijä luonnossa, sillä saalistajien on osoitettu kohdistavan valintaa tiettyjä käyttäytymisfenotyyppejä vastaan. Ei ole kuitenkaan helppoa osoittaa, että tietyt käyttäytymispiirteet ovat sopeumia erilaisiin saalistuspaineisiin, sillä käyttäytymispiirteet usein korreloivat keskenään muodostaen monimuuttujafenotyyppejä ja käyttäytymisen fenotyyppinen joustavuus on huomattavaa.

Tässä tutkielmassa tarkastelin, kuinka predaatio muokkaa kymmenpiikin (*Pungitius pungitius*) geneettisesti määräytyvää käyttäytymistä populaatiohistoriallisen predaatioriskin sekä fenotyyppisen joustavuuden kautta. Tämä saavutettiin kasvattamalla 65 täyssisarusperhettä kymmenpiikkejä, jotka olivat peräisin neljästä meripopulaatiosta (petokalasympatrinen) ja neljästä lampipopulaatiosta (ei petokaloja) common garden -asetelmassa ja mittaamalla niiden käyttäytymistä laboratoriossa luonnollisten saalistajien läsnä ollessa ja ilman. Koska tutkimuksessa käytetyt kalat olivat F1-sukupolven jälkeläisiä keinotekoisista risteytyksistä, pystyin arvioimaan myös tutkittujen käyttäytymispiirteiden heritabiliteetin ja geneettiset korrelaatiot.

Lampien kymmenpiikit olivat taipuvaisempia tutkimaan uutta ympäristöä ja ottamaan enemmän riskejä ravinnon hankkimiseksi kuin mereiset kymmenpiikit saalistusriskistä riippumatta. Sekä lampi- että merikymmenpiikit ottivat vähemmän riskejä petojen läsnä- kuin poissaollessa, mutta petojen läsnäololla ei ollut vaikutusta taipumukseen tutkia uutta ympäristöä. Koska kalat kasvatettiin yhteisessä laboratorioympäristössä, näillä populaatioeroilla on geneettinen perusta. Molemmat käyttäytymispiirteet olivat periytyviä kaikissa populaatioissa.

Tässä tutkimuksessa havaitsin geeniperustaisia ja periytyviä käyttäytymiseroja lampien ja meren kymmenpiikkien välillä. Huolimatta siitä, että käyttäytymisen joustavuus oli samankaltaista kuin merikymmenpiikeillä, lampien kymmenpiikit olivat silti huomattavan aktiivisia saalistajien läsnä ollessa, ja niillä olisi matala eloonjäämisen todennäköisyys petokalasympatrisessa ympäristössä. Taipumuksessa ottaa riskejä ravinnon hankkimiseksi välittömän predaatioriskin ja populaatiohistorian vaikutus oli samansuuntainen, mikä viittaa siihen, että ero meren ja lampien välillä tässä piirteessä johtuu erilaisista saalistuspaineista. Nämä tulokset tarjoavat todisteita käyttäytymisen paikallisesta sopeutumisesta erilaisiin saalistusasteisiin näissä populaatioissa, ja että tämä sopeutuminen tapahtuu eroina käytöksen yleisessä tasossa ennemmin kuin fenotyyppisen joustavuuden suhteen.

Avainsanat – Nyckelord – Keywords
Käyttäytymisen evoluutio, paikallinen sopeutuminen, käyttäytymisen joustavuus, käyttäytymissyndroomat, kvantitatiivinen geneetiikka, *Pungitius*, kymmenpiikki

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INTRODUCTION

Studying behaviour to understand natural selection in the wild

The study of behaviour is interesting from an ecological and evolutionary perspective, since behaviour links the neuroendocrine and structural properties of an animal to its biological functions (such as foraging and reproduction) and fitness (Réale *et al.*, 2007). In other words, behaviour determines how genetically determined physiological traits of an animal interact with the environment.

Classical behavioural ecology studies have proposed the so-called "optimality approach" (Parker and Smith 1990) wherein different individuals are expected to express optimal behaviours in different situations. The underlying assumption is therefore, that individuals would be able to express an optimal behaviour – e.g. maximizing survival or resource intake – in a given situation. Following this assumption, most of the behavioural variation observed in a natural population would be attributable to within-individual plasticity (Dingemanse *et al.* 2010). However, it is well known that individuals from a wide range of species display consistent individual differences in behaviour over time and contexts (Dingemanse and Réale, 2005; Réale *et al.*, 2007; Bell, Hankison and Laskowski, 2009; Sih and Del Giudice, 2012; Sih, Sinn and Patricelli, 2019). Behaviour within a natural population may for instance vary along the bold-shy continuum (Wilson *et al.*, 1993; Réale *et al.*, 2007) with individuals being more cautious than others displaying high levels of risk taking across different situations (Ioannou *et al.*, 2008). In a study on the rainbow trout (*Oncorhynchus mykiss*), Wilson and Stevens (2005) showed that individuals from a natural population rank differently along the bold-shy axis and that rank positions can be maintained across different contexts. The last two decades of behavioural ecology studies have brought ample evidence that consistent individual differences in behaviour – referred to as animal personality (Dall *et al.* 2004; Dingemanse *et al*. 2010) – are pervasive in the wild and explain a significant proportion of phenotypic variance in behaviour (Dingemanse *et al.* 2010).

Animal personalities have also been shown to have a genetic basis in many taxa, with natural populations housing genetic variation for behavioural traits. Specifically, quantitative genetic approaches applied to the study of behavioural traits have provided means to partition the sources of phenotypic variance (V_P) into its genetic (V_G) and non-genetic components (i.e. environmental variance, V_E; Falconer and McKay, 1996, Lynch and Walsh, 1998). By rearing related individuals in

a shared controlled environment – i.e. a common garden – and measuring behavioural variation among them, behaviour geneticists have been able to estimate the heritability (H^2) of behaviours, that is, the ratio of genetically based variation to the total phenotypic variation as per the equation:

$$H^2 = V_G / V_P.$$

In an experiment conducted with the spider *Nuctenea umbratica*, Kralj-Fišer *et al.* (2019) reared individuals in a common garden setting and estimated that 36.4% of the phenotypic variance in aggression and 22.5% of the phenotypic variance in activity were explained by genetic variation. Heritability of behavioural traits have been estimated in many other taxa and many different behaviours; for example, exploration in the great tit *Parus major* (Drent *et al.*, 2003), parental feeding effort in the long-tailed tit *Aegithalos caudatus* (MacColl and Hatchwell, 2003), antipredator behaviour in the dumpling squid *Euprymna tasmanica* (Sinn *et al.*, 2006), exploration in the poeciliid fish *Brachyraphis episcopi* (Brown *et al.*, 2007), and exploration and boldness towards predators in the three-spined stickleback *Gasterosteus aculeatus* (Dingemanse *et al.*, 2009).

Furthermore, variation in behavioural traits have also been shown to have fitness consequences (Dingemanse and Réale, 2005; Smith and Blumstein, 2008). For example, in brown trout *Salmo trutta*, variation in swimming behaviour was found to be associated with survival probability, with less active individuals suffering higher mortality (Adriaenssens and Johnsson, 2013). Similarly, behaviours related to exploration and risk-taking (i.e. "bold" behaviours) seem to be negatively associated with survival probability and life span, but positively associated with reproductive success (meta-analysis: Smith and Blumstein, 2008). The fitness effects of behavioural variation can be better understood in the context of between-species interactions, and particularly between predators and prey. The role of predation in the evolution of behaviour is important, since predators have been shown to select against particular behavioural phenotypes. In a study on anole lizards, exploratory behaviour was favoured by selection when predators were absent, whereas spending less time exposed on the ground was favoured when predators were present (Lapiedra *et al.*, 2018). In a study by Hulthén *et al.* (2017), roach (*Rutilus rutilus*) classified as bold were shown to have a higher probability to be eaten by the Great Cormorant (*Phalacrocorax carbo*), suggesting that predation by this species selects against boldness. In trout, domesticated strains that showed more risk-taking behaviour experienced lower survival than the more cautious wild strains when predation risk was high, but a higher survival when predation risk was low (Biro *et al.*, 2004). Since the observed survival cost of boldness seems to be caused by predation, the expectation is that bold behaviour would be favoured in populations with low predation risk because of the positive association between

boldness and reproductive success (Smith and Blumstein, 2008). Therefore, populations that have been locally adapted to predator-free conditions are expected to display behaviours that maximise competitive ability, growth and reproduction, but are poorly equipped to coexist with predators.

Behavioural variation is ubiquitous in wild populations and is a key component of ecological interactions between individuals and their conspecifics (e.g. Pröhl, 2003; Shine, 2003), other species (e.g. Bell and Sih, 2007; Hulthén *et al.*, 2017) and their environment (e.g. Heggenes *et al.*, 1993). Because individual behaviours are heritable and can have direct fitness consequences via their effects on survival (such as antipredator behaviour; e.g. Sinn *et al.*, 2006) or reproduction (such as courtship behaviour; e.g. Pröhl, 2003), natural selection is expected to favour evolution of different behavioural traits in different contexts. The study of behaviour and its role in adaptation is therefore central in our understanding of natural selection, and how evolutionary forces shape phenotypic variation in the wild.

Evolution of multivariate behavioural phenotypes

Natural selection seldom acts on single independent traits but rather on suites of correlated traits constituting multivariate phenotypes (Lande and Arnold, 1983; Walsh and Blows, 2009; Dochtermann and Roff, 2010). Correlated traits may not be free to evolve independently, so that evolution of one behavioural trait can be facilitated or constrained by others (Agrawal and Stinchcombe, 2009). Suites of correlated behaviours are known as behavioural syndromes (Sih, Bell and Johnson, 2004; Bell, 2007). In juvenile brown trout, exploration and activity level have been found to be correlated, thus forming an exploration-activity syndrome (Adriaenssens and Johnsson, 2013). In the field cricket *Gryllus integer* exploration and antipredator response form a behavioural syndrome that persists across different populations (Royauté *et al.*, 2019).

Behavioural syndromes raise important questions about the evolution of behaviour and behavioural geneticists have formulated two main hypotheses to explain their existence. Firstly, the so-called "constraint hypothesis" (Bell, 2005) suggests that correlated behavioural traits may not be able to evolve independently, so that evolutionary change in one trait would result in changes in another correlated trait. Alternatively, behavioural correlations may result from adaptation to an environment where certain combinations of behaviours are advantageous – the "adaptive hypothesis" (Bell, 2005). The adaptive hypothesis predicts that particular behavioural syndromes should be found only in environments where they are adaptive, and that the behaviours could be uncoupled in environments

where they are not. On the other hand, the constraint hypothesis predicts that behavioural syndromes should be ubiquitous in populations of a particular species regardless of different selection pressures. Support for the constraint hypothesis has been found at least in one study on the exploration – antipredator response syndrome of the cricket *Gryllus integer* (Royauté *et al.*, 2019), whereas many studies on the three-spined stickleback (*Gasterosteus aculeatus*) support the adaptive hypothesis with behavioural correlations being present only in populations with higher predation pressure (Bell, 2005; Dingemanse *et al.*, 2007).

Predation can drive the formation of behavioural syndromes if predators select against particular combinations of behaviour. There is indeed evidence for this; in an experiment where three-spined stickleback were exposed to actual predation, selection favoured shy and aggressive sticklebacks, while bold and unaggressive individuals showed the highest mortality (Bell and Sih, 2007). Additionally, predation generated behavioural correlations between boldness and aggressiveness in the survivors, possibly due to the removal of the most unfit bold and nonaggressive individuals from the population (Bell and Sih, 2007). Similar results were found in another study on brown trout, where boldness-aggressiveness-activity correlations strengthened in recaptured survivors (Adriaenssens and Johnsson, 2013). Since behaviours form multivariate phenotypes, the organisation of which is influenced by selection, it is important to account for behavioural correlations when studying behavioural evolution.

Behavioural plasticity

The existence of animal personalities implies that within-individual behavioural variation is limited, and individuals do not express the whole possible range of behaviour present in the population (Dingemanse et al., 2010). Nonetheless, empirical work suggests that behavioural variation in the wild is influenced by environmental factors, and that some behavioural traits display notable phenotypic plasticity in response to environmental changes (e.g. Brown *et al.*, 2007; Dingemanse *et al.*, 2012; Orci *et al.*, 2016; Dingemanse *et al.*, 2019). Phenotypic plasticity is the propensity of a genotype to express different phenotypes in different environments (West-Eberhard, 1989). Phenotypic plasticity is a central concept in evolutionary biology, and its role in the response of individuals to environmental fluctuations, and, ultimately, adaptation to changing environments has been largely integrated to the modern synthesis (e.g. Pigliucci, 2005, 2009; Merilä and Hendry, 2014).

Behavioural plasticity can be classified into two types, developmental and activational behavioural plasticity (Snell-Rood, 2013), the latter also called behavioural flexibility (Forsman, 2015). Developmental plasticity is the ability of the genotype to adopt different developmental trajectories in different environments; in behaviour, this could refer to differential brain development or learning (Snell-Rood, 2013). Activational plasticity is the differential activation of the underlying neural network in different environments that allows an immediate adjustment to the environment and the expression of different phenotypes during the animal's lifetime (Snell-Rood, 2013). Both types of plasticity are related; for example, plastic developmental changes such as learning and greater neural investment allow a greater neural innovation rate, resulting in greater potential for activational plasticity (Snell-Rood, 2013).

Behavioural plasticity can also evolve, and there may be variation in behavioural plasticity between individuals or populations (Dingemanse *et al.*, 2010). There is evidence of variation in plasticity at the species level; in the study by Sih *et al.* (2003), predator-sympatric streamside salamander species (*Ambystoma barbouri*) showed greater behavioural plasticity than the predator-naïve sister species (*Ambystoma texanum*), which was explained by differing selection pressures on plasticity related to predation. Since plasticity is known to be central in tracking rapid environmental changes (Sih *et al.*, 2011), higher levels of plasticity would be expected in more variable and unpredictable environments. A greater neural investment and resulting greater activational behavioural plasticity can be seen as an adaptation to a more variable environment (Snell-Rood, 2013). While behaviour is not infinitely plastic, it still displays notable plasticity, which need to be addressed when studying it. What is especially prominent in behavioural traits is their great potential for activational plasticity.

Challenges in the study of behavioural evolution

Since behaviours evolve through natural selection, it could be expected that adaptation to different environments would result in behavioural differences between populations of a species. However, certain aspects of behavioural traits can complicate the study of their evolution. Measuring behaviour can be difficult, since as opposed to morphological measures, behavioural measures essentially draw inferences of ecologically important traits rather than measure them directly. Therefore, special care should be applied when designing behavioural measures and interpreting results obtained by them.

Correlations between behaviours may result in individuals expressing suboptimal behaviour when a behaviour is studied in isolation, and therefore, fully understanding a behaviour may require studying

potentially correlated traits together (Sih *et al.*, 2004). For example, in a study on mole salamander (*Ambystoma* spp.) larvae, conflicting selection pressures combined with behavioural correlations across contexts resulted in all individuals displaying non-optimal behaviour in some context, such as inappropriately high exposure when predator cues were present or low exposure and feeding rates when predator cues were absent (Sih *et al.*, 2003). A significant genetic correlation between two traits provides evidence that the traits have evolved together. Most studies on behavioural syndromes only report phenotypic correlations, which do not offer robust evidence for evolutionary trade-offs (Dochtermann and Roff, 2010). Therefore, it is important to consider the multivariate nature of behaviours when studying them.

Behavioural traits display substantial phenotypic plasticity, which makes detecting inherent differences between populations difficult, and they are also often correlated with each other forming complex multivariate phenotypes. Therefore, it is important to apply methods that can disentangle genetic variance from environment-induced variation and address multivariate phenotypes. To study adaptation, replicated samples are needed from populations that experience contrasting environments, and the phenotypic variance in these populations needs to be partitioned to its genetic and environmental components. Quantitative genetic methods widely used in other areas of evolutionary biology are well-suited to addressing these kinds of problems in the study of behaviour (Dochtermann and Roff, 2010). Applying a common garden design, in which individuals originating from different populations are reared in standardised conditions, together with mixed model approaches allows the estimation of the heritabilities, genetic variances and genetic correlations among behavioural traits (Dochtermann and Roff, 2010; Dingemanse and Dochtermann, 2013).

Finally, obtaining sample sizes that are large enough to apply quantitative genetic methods can be challenging, especially in behavioural studies. Thus, few studies have estimated the heritability of behavioural traits or genetic differences in behavioural traits between populations. In order to gain strong evidence of behavioural population differences as evolutionary adaptations, quantitative genetic analyses based on large samples are needed. Answering these questions requires access to a model organism that can be found in multiple populations in contrasting environments, is easy to rear and study in a laboratory and whose biological features are suited to the study of adaptation.

Fennoscandian nine-spined sticklebacks as a model to study local adaptation

The nine-spined stickleback (*Pungitius pungitius*) is a small teleost fish, widely distributed in the circumpolar region. In recent years, the species has increased in popularity as a model organism for evolutionary, genetic and behavioural research (Merilä, 2013). The biological features of the nine-spined stickleback are well suited to the study of local adaptation.

In Fennoscandia, nine-spined sticklebacks naturally occur in the marine environment as well as in freshwater habitats such as rivers, lakes and small isolated ponds (Merilä, 2013). Pond and marine sticklebacks in this region display notable genetic and phenotypic differentiation in morphology and behaviour. There are obvious ecological differences between these environments: many freshwater ponds house no natural piscine predators of the sticklebacks whereas marine populations are sympatric to a multitude of predatory species. Therefore, the phenotypic differences between pond and marine nine-spined sticklebacks likely reflect adaptations to differing levels of predation risk (e.g. Herczeg, *et al.*, 2009, 2010; Välimäki and Herczeg, 2012).

Behaviourally, pond nine-spined sticklebacks are more explorative and aggressive, and take more risks to obtain food than marine sticklebacks (Herczeg *et al.*, 2009; Herczeg and Välimäki, 2011), which can be seen as an adaptation to a predator-free environment. This is further illustrated by the fact that pond populations of nine-spined stickleback have higher growth rates than marine populations both under and in the absence of predation threat (Välimäki and Herczeg, 2012) and display a reduced anti-predator apparatus (body armour and pelvic spines), hinting towards predation as the main driver of divergence between these populations (Herczeg *et al.*, 2009, 2010). Strong evidence for these differences being due to local adaptation to different levels of predation is, however, lacking.

Evidence for adaptive behavioural correlations have been suggested in the related species *Gasterosteus aculeatus* (three-spined stickleback), with correlations between behavioural traits only being present in populations with higher predation risk (Bell, 2005; Dingemanse *et al.*, 2007). The only study on nine-spined sticklebacks that addressed this question did not find a similar trend, possibly due to a small sample size (Herczeg, Gonda and Merilä, 2009). Thus, it is unclear whether the organisation of behavioural syndromes in these species is similar.

Pond and marine nine-spined sticklebacks have been shown to have a similar developmentally plastic behavioural responses to predators (Herczeg and Välimäki, 2011), but it is not confirmed how predation risk affects the activational component of behavioural plasticity in these populations. Even if pond sticklebacks exhibit average behaviour that would make them coexist poorly with predators, they might still have retained an activational response to predators (see Foster and Baker, 2019). Alternatively, if activational plasticity in behaviour is costly, the response may have been inhibited or lost entirely in pond sticklebacks.

Objectives

There has been increasing interest in uncovering the adaptive basis for the persistence of behavioural variation in animals, in contrast to assuming inter-individual differences to be only non-adaptive variation around an adaptive mean (Dall *et al.*, 2004). Given the stark historical, ecological and behavioural differences among wild nine-spined stickleback populations, this species represents a particularly opportune model to address the role of behaviour in local adaptation.

The aim of the present study was to investigate whether pond and marine nine-spined sticklebacks have locally adapted to different levels of predation in behavioural traits. I also explored possible differences in activational plasticity and behavioural correlations between these populations. Additionally, I aimed to estimate the heritability of behaviours and possible differences in levels of genetic variation in behaviours between large (marine) and small (pond) populations. These questions were approached by studying two ecologically important behavioural traits: exploration of a novel environment and risk-taking during foraging, in laboratory-born, first generation individuals in the presence and absence of natural predators.

First, I hypothesized that pond sticklebacks would be more explorative and take more risks during foraging both in the presence and absence of predators than marine sticklebacks. Because pond sticklebacks have evolved in an environment free of piscine predators for a long time, being cautious would not improve survival and would rather be disadvantageous in competitive situations. Second, I expected marine populations to exhibit a stronger activational plastic response to predators than pond populations due to the overall higher complexity of the marine habitat and higher level of genetic variation in marine populations. Third, I hypothesised that marine populations would exhibit phenotypic and genetic behavioural correlations (behavioural syndromes) in contrast to pond populations due to an adaptive behavioural syndrome, and that these correlations would tighten in the

presence of predators. Finally, I expected both behaviours to be heritable in all populations, and that the amount of quantitative genetic variation in behaviour is positively correlated with the genetically effective population size of the focal populations.

MATERIALS AND METHODS

Sampling and rearing

Adult *P. pungitius* were sampled during breeding season (May – June 2018) at eight different locations in Sweden and Finland corresponding to four coastal marine and four freshwater pond habitats (Fig. 1). Pond populations were sampled using minnow traps placed in ca. 50 cm depth and marine populations were sampled from shallow (ca. 1-meter depth) waters using beach-seine nets. Sampled fish were checked visually to ensure sexual maturity (i.e. black abdomen in males and gravid females, e.g. McLennan, 1996) and subsequently transported to the aquaculture facilities of the University of Helsinki. Wild-caught individuals from each population were housed separately in $1m^3$ plastic aquaria with flow-through water system and fed *ad libitum* with frozen chironomid larvae twice a day.

For each population, five to ten full-sib families were produced ($n = 65$; Table 1) by artificial crossing of wild-caught individuals. The standard split-clutch *in vitro* fertilization techniques and egg husbandry protocols for stickleback crossing were followed (Barber and Arnott, 2000) and eggs were obtained from gravid females by gently squeezing their abdomens over a petri dish. Males were over-anesthetized using tricaine methane sulfonate (MS-222) in order to extract their testes, which were subsequently minced in the petri dish containing the eggs. Eggs and sperm were mixed using a plastic pipette to ensure fertilization and kept in water until hatching. Water in the petri dishes was changed twice a day and clutches were visually checked for signs of fungal infections or death, and accordingly removed. At the onset of hatching and for a four weeks period, each clutch was split in two replicate 11 x 10 cm plastic boxes. Following yolk resorption, fry were fed *ad libitum* with live brine shrimp (*Artemia sp.* nauplii).

All family replicates were transferred to Allentown Zebrafish Rack Systems (hereafter rack, Aquaneering Inc., SanDiego, USA). Racks had a closed water circulation system, with multi-level filtering including physical, chemical, biological and UV filters. All fish were reared under constant temperature and light conditions (15°C; 12:12 LD) until the start of the behavioral experiment.

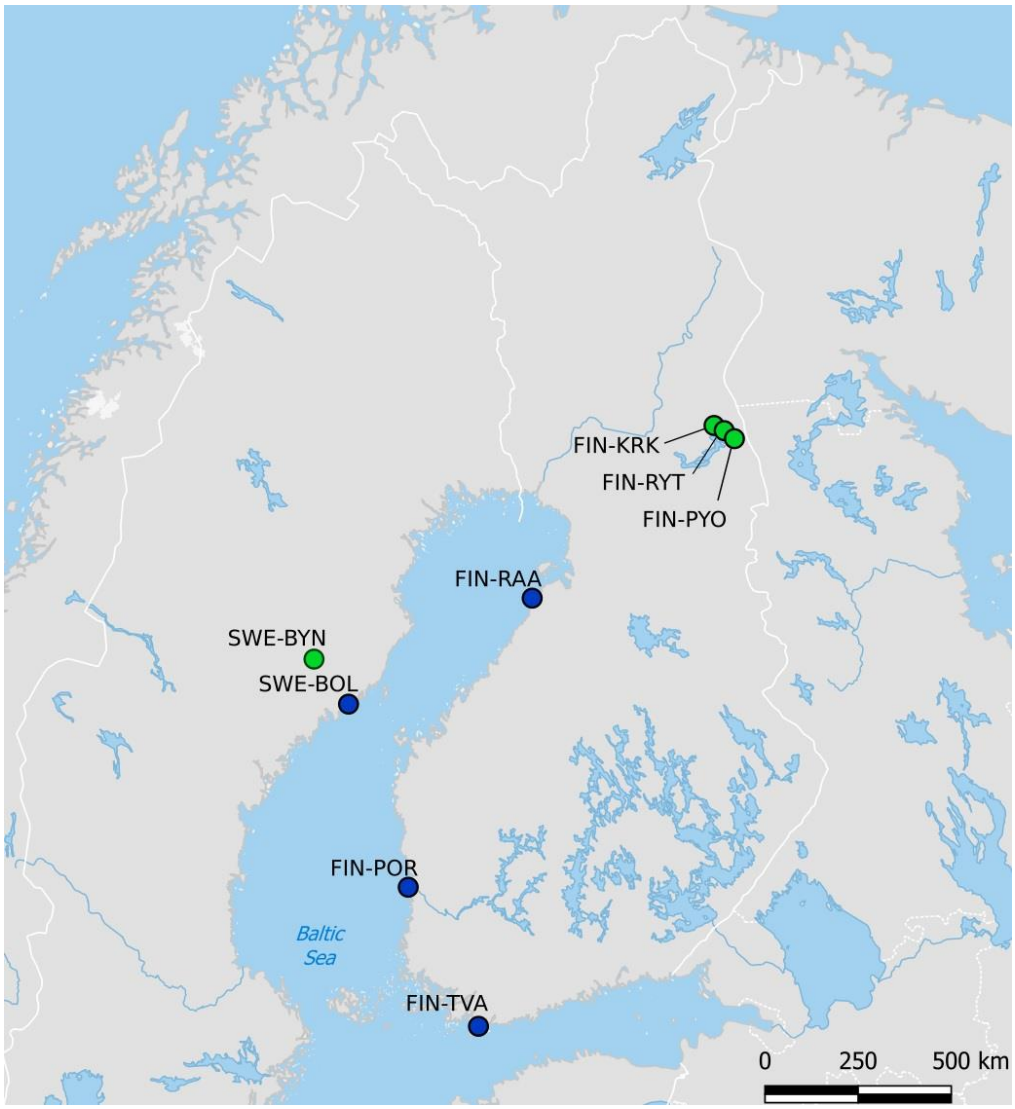

Fig. 1. Locations of the sampling sites. Freshwater ponds are marked with green and marine locations with blue circles.

Table 1. Numbers of full-sib families and individuals used in the present study.

Habitat	Population	Abbreviation	No. of families	Total no. of individuals assayed
pond	Bynastjärnen, SWE	BYN	10	46
	Kirkasvetinenlampi, FIN	KRK	5	30
	Pyöreälampi, FIN	PYO	5	49
	Rytilampi, FIN	RYT	10	76
	Total		*30*	*201*
marine	Pori, FIN	POR	10	71
	Raahe, FIN	RAA	5	40
	Tvärminne, FIN	TVA	10	75
	Bölesviken, SWE	BOL	10	74
	Total		*35*	*260*
Total			***65***	***461***

Experimental procedure

Before starting the experiments, all families were transferred to holding tanks where they were kept in constant temperature and light conditions (15°C; 12:12 LD) throughout the experimental period. Replicates of the same family were housed separately in order to account for common environment variance.

Experimental aquaria

Two identical experimental aquaria with independent flow-through water systems were built for the experiments (Fig. 2). Each aquarium was divided transversely in two sections by a transparent plastic plate separating the behavioural arena and the holding arena. The behavioural arena corresponded to the half of the tank were the focal stickleback fish were placed and scored for behaviours, while the holding arena corresponded to the half were the predators were introduced. Behavioural arenas were lined with polystyrene to prevent any visual disturbance from outside. Both aquaria had a clump of artificial algae (used as a refuge) and an opaque plastic cylinder with a small openable door in another corner against the same wall as the refuge (Fig. 2 and see the *Exploration* section below). A piece of wire was attached to the inner cylinder, so that it could be lifted without touching the cylinders.

Predation and control treatments

In order to investigate the effect of predation risk on stickleback behaviour, behavioural tests were conducted in the presence and absence of predators. One of the experimental aquaria (see above) was assigned to predation treatment and one to control treatment.

In the predation treatment, a pair of wild-caught perch (*Perca fluviatilis)*, a natural predator of sticklebacks (both in sea and freshwater), were placed on the holding arena of the experimental aquarium. The water flowed from the holding arena to the behavioural arena, so that focal fish in the predation treatment could get chemical and visual cues from the perch. The perch were changed six times during the experimental period to prevent pseudoreplication as well as habituation of the perch to the stickleback stimuli. A total of nine perch and seven unique perch pairs were used in the study.

In the control treatment, the experimental aquarium housed no perch in the holding arena, so that the space behind the transparent divider was empty. The water in the control and predation treatment

aquaria was not connected in any way so that fish in the control aquarium could not get any chemical nor visual cues from the perch. Apart from the absence of perch, the experimental aquarium was strictly identical to the one used in predation treatment.

Behavioural tests

I measured two categories of ecologically relevant behaviour: exploration (an individual's latency to start exploring a novel environment; following Herczeg, *et al.*, 2009), and risk-taking during foraging (an individual's tendency to take risks to obtain food). The behavioural tests were performed with one fish at a time. Fish were starved for 24 hours before the behavioural experiments. Each trial started by introducing the focal fish into the experimental tank and running the exploration test followed by the risk-taking test (detailed below). All behavioural testing was conducted over the course of 37 days in April-May 2019 divided to two temporal blocks, morning (8:30 – 12:30) and afternoon (13:00 – 18:00). At the time of the testing, the mean age of the fish was 316.4 days with a standard deviation of 23.8 days.

Since expression of exploration is known to differ between socially and solitarily reared fish (Jolles, Aaron Taylor and Manica, 2016), replicates that had only a single fish were not assayed, so that all fish used in the experiments had been reared in a group. From each family, eight individuals were used for the experiments. If a family had less than eight fish, all individuals were used. Within each family, individuals were distributed evenly between treatments (predation and control, see above) and temporal blocks (morning and afternoon), so that each group had representation from both of the replicates in a family. If the family had less than eight individuals, individuals were assigned into groups prioritising even distribution among (i) treatments, (ii) replicates, and (iii) temporal blocks. All individuals were distributed in a random order across the experimental days.

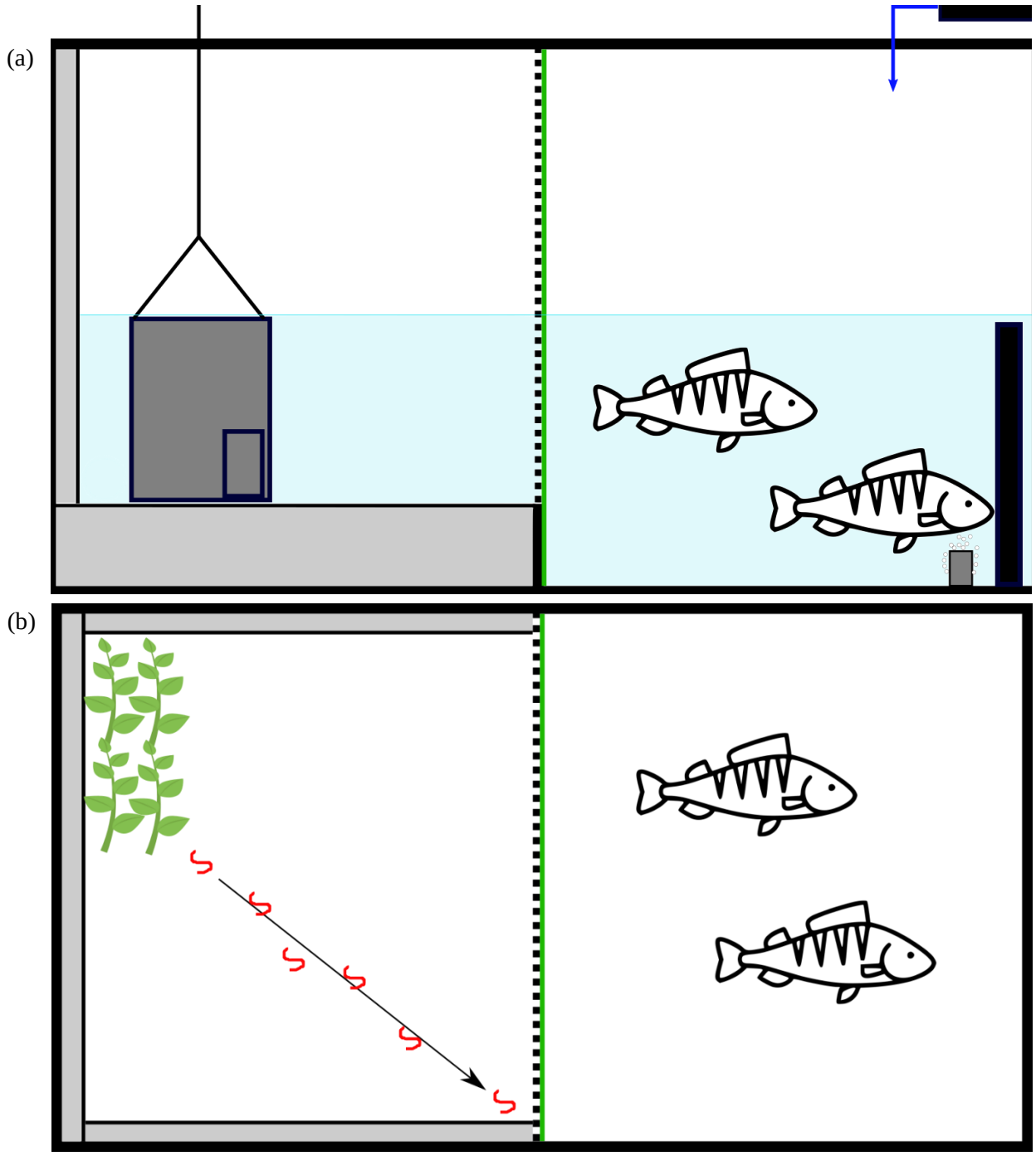

Fig. 2. Schematic representation of the experimental aquaria. (a) Lateral view of the experimental aquarium during the exploration test. The left side corresponds to the behavioural arena including the polystyrene lining (grey rectangles) and the opaque plastic cylinder used for exploration trials. The right side corresponds to the holding arena housing the pair of perch (for predation treatment), the water inlet (blue arrow) and outlet (black rectangle) as well as an air stone (grey rectangle). (b) Top view of the experimental aquarium in the risk-taking test . A clump of artificial algae was set up as a refuge in the behavioural arena (top left corner). Chironomid larvae (food item, in red) were pipetted into the tank following a diagonal line (black arrow). A transparent non-hermetic plastic divider (transversal green and dashed black lines) was set up to allow visual and chemical cues between behavioural and holding arenas. Perch were introduced in the holding arena (right side) in the predation treatment only.

Exploration

Exploration was tested in terms of individual latency to start exploring a novel environment. The focal fish was caught from its holding tank with a hand net and introduced into the cylinder in the experimental tank (see Fig. 2a). The fish was left to acclimatise inside the cylinder for three minutes. After this acclimation time the door of the cylinder was opened allowing the fish to leave the cylinder to explore the experimental tank.

Two measurements were recorded: the latency (in seconds) until the head of the fish came out of the cylinder, and the latency (in seconds) until the full body of the fish came out of the cylinder. The experiment was terminated if the fish did not appear after five minutes, so that the maximum value for both measurements was 300 seconds.

Risk-taking during foraging

Following the exploration test, the cylinder was removed, and the fish was left to acclimatize for three minutes in the behavioural arena. After the acclimation period, chironomid larvae (a familiar food) were pipetted into the open area of the tank in a straight diagonal line from the edge of the refuge to the opposite corner of the tank (see Fig. 2b). With this kind of food administration, the more the fish ate, the further it had to move from the refuge, so that the "risk" experienced by the fish (swimming further into the open area and closer to the predator) was proportional to the "reward" (number of food items).

Three measurements were recorded: the time the fish spent in the open area (whole body outside the refuge area when viewed from above) in the five minutes following the addition of the first larvae (in seconds); the latency to first feeding after the addition of the first larvae (in seconds); and the total number of feeding events calculated as the number of successful attacks on the food. The experiment was terminated after five minutes, so that the maximum values for the latency to first feeding and for time in the open area were 300 seconds.

Morphological measurements

After the behavioural tests, fish were euthanized with an overdose of MS-222. The fish were then photographed and preserved in ethanol. Standard length (body length measured from the tip of the

snout to the posterior end of the hypural plate, i.e. excluding the length of the caudal fin) was measured from the photographs using the imageJ software (ver. 1.52; Rasband, W.S., ImageJ, U. S. National Institutes of Health, 2018).

Ethical statement

All experiments were conducted under a permit from the Animal Experiment Board in Finland (permit reference ESAVI/4979/2018).

Statistical analyses

Data handling

All time-based variables (latency to head out, latency to body out, time in the open area, latency to first feeding) were strongly bimodal, with most individuals having "extreme" phenotypes and with very few intermediate phenotypes. To explore the effects of habitat, population and treatments on behavioural traits, the raw variables were transformed into two datasets used subsequently for data visualization and group comparisons, and to run generalized linear models to test for their statistical significance.

First, the time-based variables were transformed into binary variables, using a 150-second (the midpoint of the possible range) cut-off point. The distributions of the two exploration variables (latency to head and body out) were nearly identical, so these variables were combined into one by taking the mean of these two variables before binary transformation. Thus, all individuals were classified as explorative or non-explorative, spending a lot or little time in the open area and being either quick or slow feeders. The proportions of explorative individuals, individuals spending a lot of time in the open area and quick feeders were compared across populations and treatments using pairwise tests for proportions with Bonferroni correction. The amount of feeding events was compared across populations and treatments using pairwise t-tests for means with Bonferroni correction.

Second, I followed the approach used in earlier studies on stickleback behaviour (e.g. Herczeg *et al.*, 2009; Dingemanse *et al.*, 2012) and used Principal Component Analyses (PCA) to collapse the set of raw behavior variables into two meaningful behavioural PC scores. Variables for exploration (latency

to head out and time to body out) and risk-taking during foraging (time in open area, latency to first feeding, number of feeding events) were collapsed together by running two PCAs across populations. For both behaviours, only the first PC had an eigenvalue above 1. For exploration, the first principal component explained 97,7% of variance in the two variables. For risk-taking during foraging, the first component explained 79,4 % of variance in the three variables. These two PC scores (hereafter $PC1_{exp}$ and $PC1_{for}$) were extracted from the analysis and used as response variables in the subsequent linear models. I examined the validity of these scales by performing an exploratory factor analysis with two factors across all populations (detailed in Supplementary methods). This analysis confirmed that the behavioural variables corresponded to the presumed two categories of behaviours.

Survival analysis

To further investigate the effects of habitat and the predation treatment on both behaviour types, I analysed the latency-based data (see above) by performing a survival analysis using the *survival* (ver. 3.2-3; Therneau, 2020) and *survminer* (ver. 0.4.8; Kassambara *et al.,* 2020) R packages. Specifically, I used the Kaplan-Meier estimator implemented in the *survival* package to create survival curves for each habitat and treatment. Although my data do not represent survival data *per se*, application of this analysis to the latency-based variables allowed to get visual representation of the proportion of individuals expressing a behaviour throughout the whole measurement period, and to see at which time point the populations started to diverge in their behavioural responses (see Results).

Generalized linear models

To compare exploration and risk-taking across habitats (pond and marine) and treatments (predation and control), I ran Markov Chain Monte Carlo general linear mixed models implemented in the *MCMCglmm* R package (ver. 2.29; Hadfield, 2010) using the two main PC scores (see above) as dependent variables. I ran the complete model for $PC1_{exp}$ and $PC1_{for}$, respectively, using all possible effects including habitat, treatment, habitat-treatment interaction and age-corrected body size as fixed effects and included replicate tank, temporal block, population of origin and the animal term (corresponding to individual identity with pedigree information) as random effects. Initial investigation of the model results showed that the effects of replicate and temporal block (see *Behavioural tests* above) explained close to zero variance. These two effects were removed from the full models. The habitat-treatment interaction was non-significant, and its exclusion improved model fit based on the Deviance Information Criterion (DIC) in both models, so it was removed from the

final models as well.

The final models included habitat, predation treatment and age-corrected body size as fixed effects, and the animal term and population of origin as random effects. Each model was run for 5 500 000 iterations with an 500 000 burn-in period and posterior samples were thinned every 5 000 iterations.

Quantitative genetics parameters

To estimate the heritability of the behavioural traits, I used the animal model approach implemented in the *MCMCglmm* R package (ver. 2.29; Hadfield, 2010). Pedigree information – i.e. the relatedness between individuals – allowed the partitioning of the phenotypic variance into its genetic and environmental components using the animal model. For each population-treatment group, I ran two models including $PC1_{exp}$ and $PC1_{for}$, respectively, as a response variable and animal term as a random effect. Each model was run for 5 500 000 iterations with an 500 000 burn-in period and posterior samples were thinned every 5 000 iterations. Broad sense heritability estimates (H^2) and their corresponding 95% highest posterior density intervals (HPDI) were obtained from the posterior distributions by solving:

$$H^2 = V_G / V_G+V_R$$

where V_G and V_R correspond to the genetic and residual components of variance, respectively.

To investigate the existence of behavioural syndromes, I calculated phenotypic and genetic correlations between exploration and risk-taking in all populations and treatments. To test for phenotypic correlations, Spearman rank correlation coefficients with Bonferroni correction were used to test the association between exploration and risk-taking within each population-treatment group. Genetic correlations were calculated by running a bivariate animal model in *MCMCglmm* for each population-treatment group including $PC1_{exp}$ and $PC1_{for}$ as response variables and animal term as a random effect.

All analyses were performed in R (ver. 3.5.3; R Development Core Team, 2019).

Estimation of historical effective population size

Finally, estimates of historical effective population size (N_e) from another study (Feng *et al.,* unpublished results) were used to investigate the correlation between N_e and quantitative genetic

variation. Briefly, the Site Frequency Spectrum (SFS) for each population was first generated from Whole Genome Sequencing (WGS) data using ANGSD (Korneliussen *et al.* 2013). Second, a neutral standard process (assuming no population size change) was modelled using ∂a∂i (Gutenkunst *et al.* 2009). From the ∂a∂I outputs, estimations of per-population N_e were recovered by solving the equation:

$$Ne = \frac{4\theta^O}{4\mu n}$$

Where θ^O corresponds to the estimator of nucleotide diversity optimized by maximum likelihood; μ is the mutation rate (μ = 1.42E-08) and n is the number of sites (n = 1000).

RESULTS

Exploration

Pond sticklebacks were more explorative than marine sticklebacks and the proportion of explorative fish was higher in pond populations regardless of the predation treatment (χ^2 = 15.1, df = 1, p < 0.001 in control treatment; χ^2 = 23.9, df = 1, p < 0.001 in predation treatment; Fig. 3a). The effect of predation on explorative behaviour was not significant in either of the two habitats (Fig. 3a).

Differentiation in explorative behaviour between habitats was expressed early on in the behavioural test, with 60 % of the pond individuals having left the refuge by 50 seconds in the control treatment, while only 43% of marine individuals had left the refuge at the same time-point in the control treatment (Fig. 4). At the end of the exploration trial in the control treatment, 89 % of the pond individuals had started to explore the tank in contrast to 68 % of the marine individuals. The difference between the survival curves of pond and marine individuals was statistically significant (log rank test; χ^2 = 31.4, df = 1, p < 0.001; Fig 4). Predation treatment did not have a significant effect on explorative behaviour in neither habitat based on the log rank test from survival curves (χ^2 = 0.1, df = 1, p = 0.7).

These results were confirmed by the *MCMCglmm* analysis using PC_{exp} extracted from all individuals, with a significant effect of the habitat of origin on exploration tendency, and pond origin being associated with higher levels of exploration (Table 2). Predation treatment had no statistically significant effect on exploration (Table 2).

Risk-taking during foraging

Marine individuals were less likely to spend time in the open area than pond individuals in both treatments ($\chi^2 = 21.9$, df = 1, $p < 0.001$ in control treatment; $\chi^2 = 43.8$, df = 1, $p < 0.001$ in predation treatment), and less likely to spend time in the open area in the presence of predators than when predators were absent ($\chi^2 = 8.5$, df = 1, $p = 0.021$). Predation treatment did not have an effect on the proportion of pond sticklebacks spending a lot of time in the open area (Fig. 3b).

Pond fish were more likely to feed quickly than marine sticklebacks in both treatments ($\chi^2 = 20.6$, df = 1, $p < 0.001$ in control treatment; $\chi^2 = 28.5$, df = 1, $p < 0.001$ in predation treatment; Fig. 3c). The presence of predators decreased the proportion of quickly-feeding individuals in both habitats ($\chi^2 = 7.74$, df = 1, $p = 0.032$ for marine individuals; $\chi^2 = 10.8$, df = 1, $p = 0.006$ for pond individuals; Fig. 3c).

Pond sticklebacks ate more food items both in the presence and absence of predators than marine sticklebacks (t = -6.04, df = 167, $p < 0.001$ in control treatment; t = -4.30, df = 144, $p < 0.001$ in predation treatment; Fig. 3d). Both marine and pond sticklebacks ate fewer food items in the presence of predators compared with the control treatment (t = 4.10, df = 193, $p < 0.001$ for marine; t = 5.43, df = 163, $p < 0.001$ for pond; Fig. 3d).

There was a strong difference in the initiation of foraging behaviour (i.e. latency to first feeding) between habitats early on in the risk-taking trial (Fig. 5). In the control treatment, 79% of the pond individuals had already initiated foraging by 50 seconds, whereas only 43 % of the marine fish had. By the end of the control trial, 87 % of pond fish had initiated feeding, while only 50 % of marine fish had eaten anything (Fig. 5). This differentiation in foraging behaviour was observed in both treatments; in the predation treatment 53% of pond fish had initiated feeding by 50 seconds, while only 18 % of marine fish had (Fig. 5). By the end of the predation trial, 67 % of pond fish and 34 % of marine fish had initiated feeding. The difference between the survival curves of pond and marine individuals was statistically significant with pond individuals being quicker and more likely to feed (log rank test; $\chi^2 = 58.9$, df = 1, $p < 0.001$; Fig. 5). Predation risk significantly increased the latency to first feeding in both habitats (log rank test; $\chi^2 = 19.3$, df = 1, $p < 0.001$).

The *MCMCglmm* analysis using PC_{for} as dependent variable confirmed that habitat of origin and predation treatment had a statistically significant effect on the tendency to take risks during foraging (Table 2).

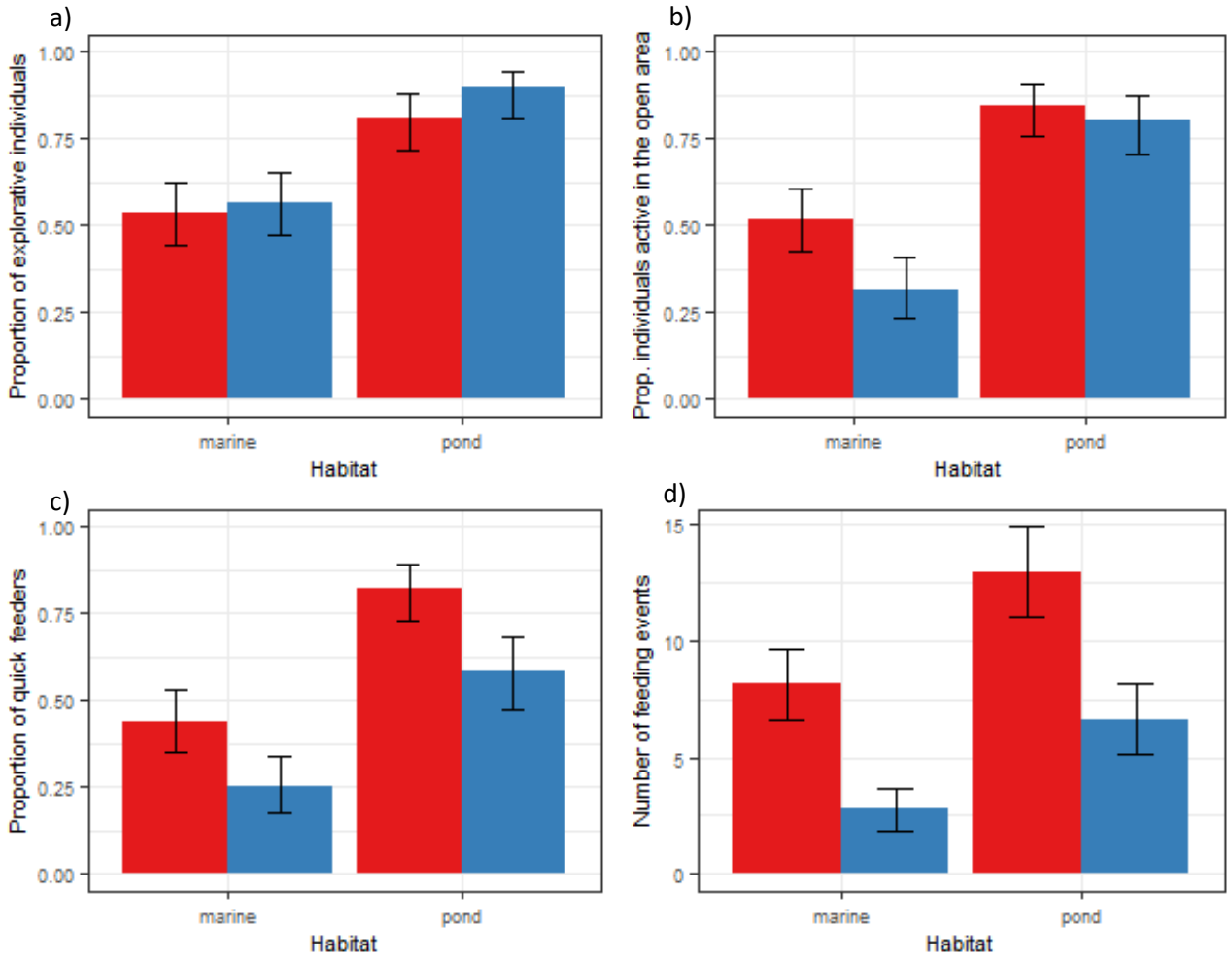

Fig. 3. **Comparison of behavioural types among habitats and treatments**. a) Proportion of individuals in each group classified as explorative in the exploration trial. b) Proportion of individuals in each group classified as spending a lot of time in the open area in the risk-taking trial. c) Proportion individuals in each group classified as quick feeders in the risk-taking trial. d) Mean number of feeding events during the risk-taking trial. Proportions are shown for each habitat (marine, pond) whether in the presence of predator (blue) or in the control treatment (absence of predator; red). Black error bars show the 95% confidence intervals.

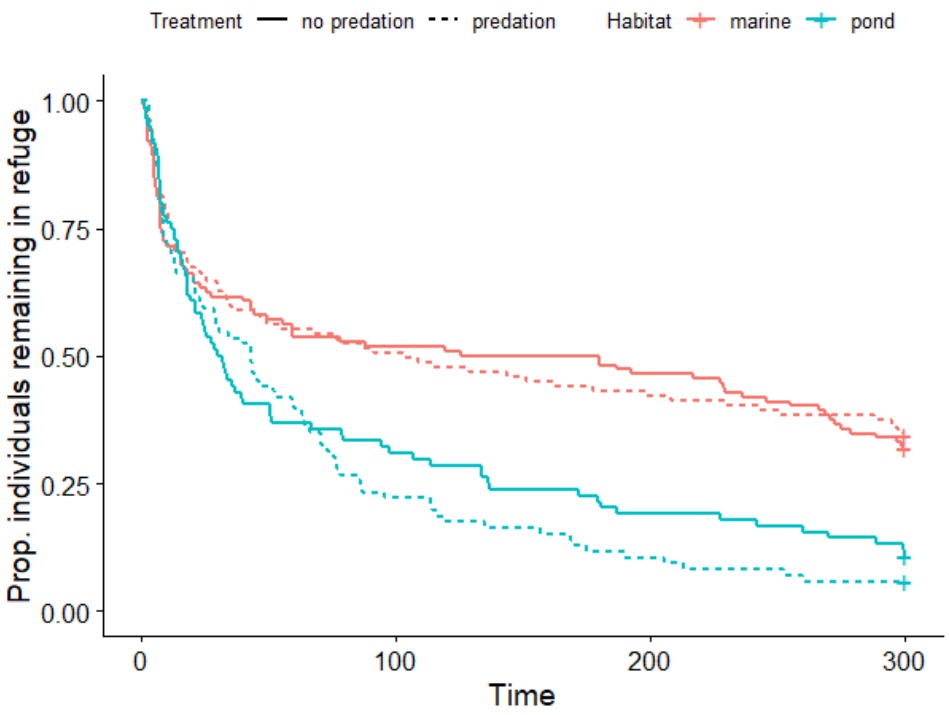

Fig. 4. Survival curves for the exploration behaviour. Proportion of assessed individuals remaining inside the refuge at each time point (in seconds) throughout the exploration trials in each habitat-treatment group.

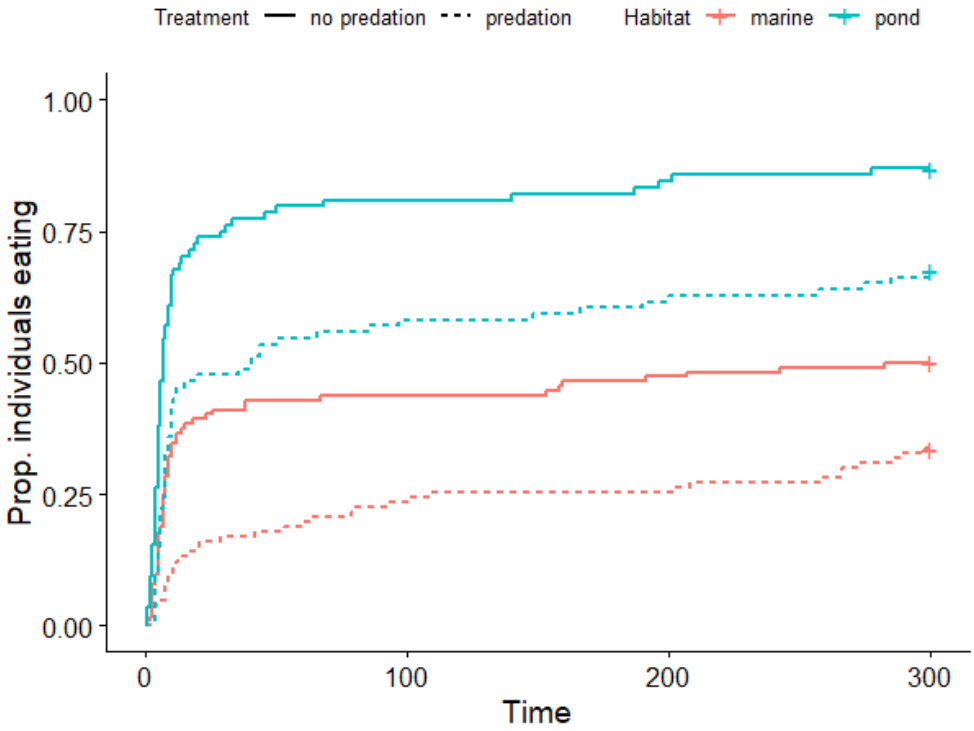

Fig. 5. Survival curves for the latency to first feeding. Proportion of assessed individuals that had started eating at each time point (seconds) throughout the foraging trials in each habitat-treatment group.

Table 2. Results of the *MCMCglmm* analyses. Posterior mean values and 95% credible intervals for the fixed effects of the MCMCglmm using $PC1_{exp}$ and $PC1_{for}$ as dependent variables. For random effects, see text.

Dependent variable	**Effect**	**Mean**	**95% credible interval**	***p***
Exploration ($PC1_{exp}$)	Intercept	-0.444	[-0.734; -0.162]	0.006
	Habitat (pond)	*0.876*	*[0.470; 1.25]*	*<0.001*
	Predation treatment	0.105	[-0.156; 0.343]	0.43
	Body length	-0.0310	[-0.0617; 0.0033]	0.064
Risk-taking during foraging ($PC1_{for}$)	Intercept	-0.192	[-0.483; 0.110]	0.21
	Habitat (pond)	*1.47*	*[1.06; 1.91]*	*<0.001*
	Predation treatment	*-0.817*	*[-1.08; -0.559]*	*<0.001*
	Body length	-0.0286	[-0.0571; 0.00470]	0.084

Behavioural correlations

In the pooled sample of all populations, exploration and risk-taking were positively correlated (Table 3). Considering both treatments together, one pond (RYT) and three marine populations (BOL, TVA, POR) displayed positive phenotypic correlations between exploration and risk-taking (Table 3). In the absence of predators, two pond (RYT, BYN) and three marine (BOL, TVA, POR) populations displayed positive phenotypic correlations, whereas in the presence of predators, only one pond (RYT) and one marine (TVA) population showed positive phenotypic correlations (Table 3).

Genetic correlations between exploration and risk-taking were non-significant in all the individual populations (Table 4). However, when pooling all the pond and marine populations together, respectively, there was a genetic correlation in both pooled pond and marine populations in the absence, but not presence, of predators (Table 4).

Table 3. Phenotypic Spearman rank correlations (r_s) between exploration and risk-taking.

Habitat	Population	Predator absence		Predator presence		Both treatments	
		r_s	p	r_s	p	r_s	p
Pond	BYN	*0.44*	*0.0406*	-0.0614	0.776	0.155	0.303
	KRK	0.204	0.466	0.358	0.19	0.205	0.277
	PYO	-0.0214	0.919	-0.125	0.561	0.0988	0.5
	RYT	*0.42*	*0.00872*	*0.321*	*0.0491*	*0.359*	*0.00147*
Marine	RAA	0.297	0.203	0.177	0.457	0.277	0.083
	POR	*0.666*	*<0.001*	0.228	0.187	*0.512*	*<0.001*
	TVA	*0.452*	*0.00491*	*0.348*	*0.0323*	*0.432*	*<0.001*
	BOL	*0.487*	*0.00167*	0.246	0.154	*0.354*	*0.00196*
All		*0.509*	*< 0.001*	*0.258*	*<0.001*	*0.397*	*<0.001*

Table 4. Genetic correlations (r_g)between exploration and risk-taking with 95 % confidence intervals.

Habitat	Population	Predator absence		Predator presence	
		r_g	CI	r_g	CI
Pond	BYN	0.581	[-0.356; 0.895]	0.322	[-0.444; 0.844]
	KRK	-0.0392	[-0.638; 0.666]	0.409	[-0.557; 0.864]
	PYO	0.279	[-0.358; 0.798]	0.169	[-0.674; 0.642]
	RYT	0.726	[-0.0354; 0.886]	0.398	[-0.311; 0.819]
	All	*0.607*	*[0.00483; 0.872]*	0.483	[-0.142; 0.816]
Marine	TVA	0.741	[-0.128; 0.923]	0.211	[-0.561; 0.794]
	POR	0.703	[-0.281; 0.941]	0.181	[-0.524; 0.821]
	BOL	0.678	[-0.139; 0.949]	0.577	[-0.511; 0.854]
	RAA	0.113	[-0.666; 0.6696]	0.491	[-0.793; 0.525]
	All	*0.598*	*[0.0117; 0.912]*	0.319	[-0.407; 0.697]

Heritability and genetic variation

Both behavioural traits were heritable in all populations and in both treatments (Table 5). There were no statistically significant differences in heritability estimates between populations or treatments.

There was no statistically significant correlation between effective population size and genetic variation (posterior mode 0.589; HPD [-0.276; 0.977]) or effective population size and heritability (posterior mode -0.283; HPD [-0.769; 0.798]). There was, however, a positive trend (albeit non-significant) for genetic variation and effective population size to be correlated (fig. 6a). The correlations between effective population size and heritability clustered around zero (fig. 6b).

Table 5. Posterior heritability estimates and 95% credible intervals.

Habitat	Population	Predator absence				Predator presence			
		Exploration tendency		Risk-taking during foraging		Exploration tendency		Risk-taking during foraging	
		H^2	CI	H^2	CI	H^2	CI	H^2	CI
Pond	BYN	0.325	[0.0657; 0.816]	0.669	[0.220; 0.914]	0.423	[0.223; 0.859]	0.305	[0.100; 0.875]
	KRK	0.574	[0.249; 0.828]	0.626	[0.160; 0.907]	0.467	[0.105; 0.884]	0.612	[0.192; 0.889]
	PYO	0.526	[0.231; 0.763]	0.439	[0.140; 0.792]	0.514	[0.250; 0.773]	0.297	[0.114; 0.848]
	RYT	0.491	[0.200; 0.837]	0.652	[0.176; 0.896]	0.324	[0.194; 0.820]	0.617	[0.189; 0.835]
	All	0.35	[0.153; 0.698]	0.551	[0.277; 0.873]	0.398	[0.256; 0.828]	0.312	[0.136; 0.754]
Marine	RAA	0.590	[0.225; 0.774]	0.449	[0.146; 0.845]	0.445	[0.212; 0.871]	0.627	[0.193; 0.863]
	POR	0.275	[0.0694; 0.811]	0.172	[0.0818; 0.845]	0.390	[0.127; 0.827]	0.308	[0.155; 0.801]
	TVA	0.666	[0.219; 0.904]	0.813	[0.271; 0.943]	0.190	[0.0866; 0.774]	0.320	[0.131; 0.740]
	BOL	0.428	[0.136; 0.893]	0.459	[0.112; 0.851]	0.531	[0.125; 0.874]	0.255	[0.149; 0.855]
	All	0.286	[0.0962; 0.645]	0.488	[0.171; 0.847]	0.305	[0.0967; 0.615]	0.301	[0.104; 0.554]

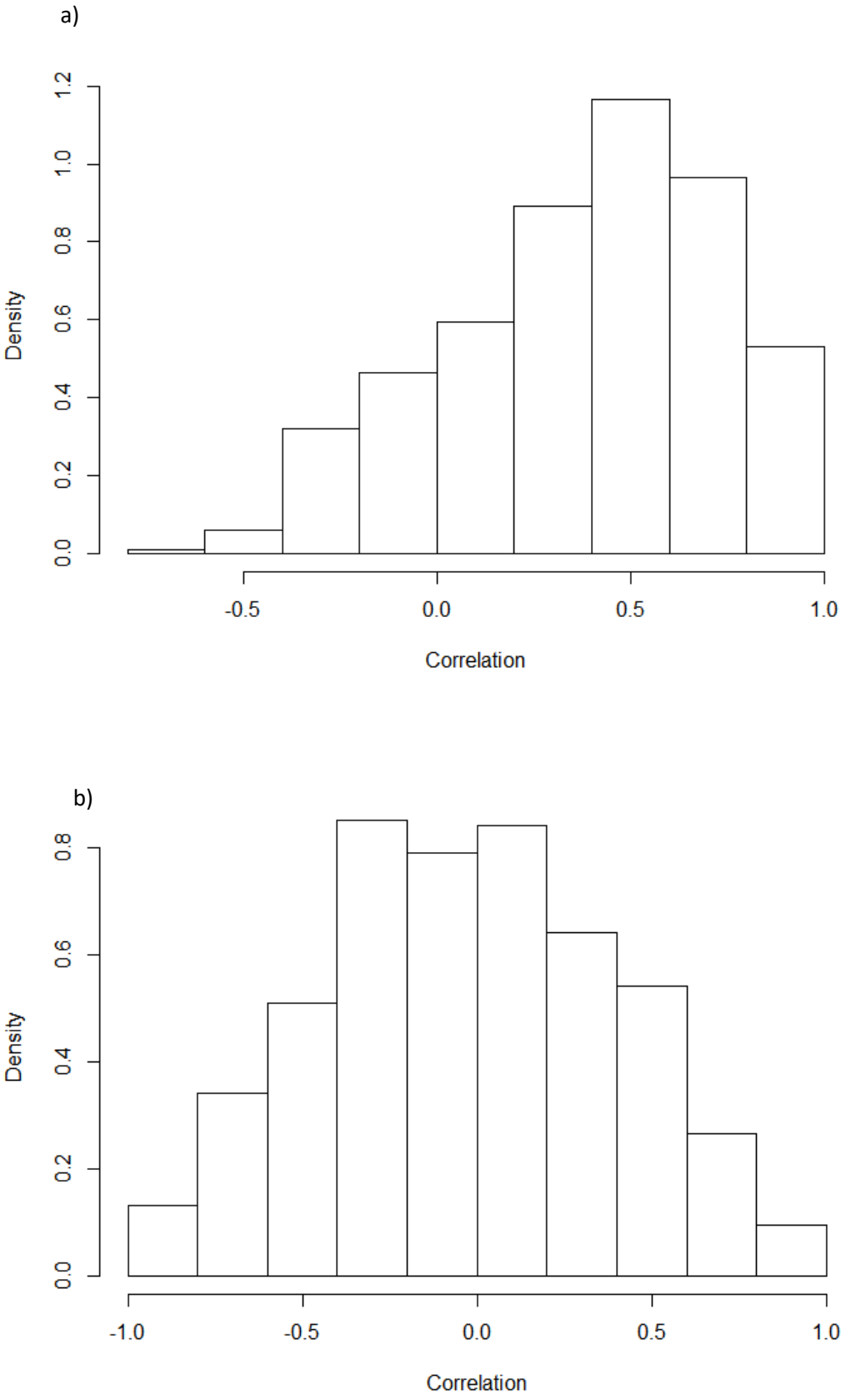

Fig. 6. **Association between genetic variance (V_G) , heritability and effective population size (Ne).** a) Distribution of correlation estimates for the correlation between genetic variance and effective population size. b) Distribution of correlation estimates for the correlation between heritability and effective population size.

DISCUSSION

Effect of population history

There were marked behavioural differences between marine and pond sticklebacks. Pond sticklebacks were more explorative and took more risks while foraging both in the presence and absence of natural predators compared to their marine conspecifics. These findings are in agreement with those found in earlier studies on nine-spined sticklebacks (Herczeg, Gonda and Merilä, 2009; Herczeg and Välimäki, 2011).

In contrast to previous studies on the behaviour of nine-spined sticklebacks (e.g. Herczeg, Gonda and Merilä, 2009; Herczeg and Välimäki, 2011; Laine *et al.*, 2014), all fish in this study were reared in groups. Since nine-spined sticklebacks display social behaviour such as schooling, it is possible that the behaviours measured here were affected by social learning. In a study by Frost *et al.* (2007), rainbow trout that observed shy conspecifics were found to become more shy, whereas observing bold conspecifics had no effect on boldness. Therefore, it is possible that shy behaviour (low exploration and risk-taking) was enforced in shy groups also in this study. This, however, might only accentuate existing behavioural differences, and would not have an effect on conclusions. This is especially the case since the bold behaviour of the pond populations would have been relatively unaffected by group rearing. Overall, the large replicated common garden design of this study provides robust evidence for the genetic basis of behavioural variation in wild stickleback populations from the two contrasting habitats.

I also observed variation in behaviours at the population level. One of the four marine populations (Raahe) showed much higher levels of exploration in both contexts than the other marine populations and a high level of risk-taking in the absence of predators (but not in the presence of predators; see fig. S1-S4). A previous study investigating variation of wild nine-spined stickleback body size, one marine population (Bölesviken) showed marine-like morphology (i.e. small size and body armour), but pond like growth responses to food and predation treatments (Välimäki and Herczeg, 2012). In my study, the Bölesviken population showed typical, marine-like behaviour. It seems that some coastal Baltic marine populations show a mixture of "marine-like" and "pond-like" characteristics, possibly due to possible differing selection pressures in these areas, or influx of freshwater fish to coastal areas. Also, one pond population (Kirkasvetinenlampi) showed very passive, "marine-like" behaviour, with

individuals being exceptionally cautious compared to individuals from other ponds. It should be noted that both of these exceptional populations had much lower sample sizes than the rest of the populations (see Table 1), and thus, these results should be interpreted with caution. Excluding these populations from the analyses did not change the conclusions but increased the difference between marine and pond populations (see fig. S5-S6).

Behavioural response to predators

Predator presence reduced risk taking during foraging, but not the tendency to explore. The response was similar across habitats, implying that pond sticklebacks have retained a similar activational plastic response to predators as the ancestral marine form. An earlier study found that marine and pond nine-spined sticklebacks have a similar developmental plastic response to predators (Herczeg and Välimäki, 2011). It seems, therefore, that behavioural plasticity has not evolved in pond nine-spined sticklebacks. Since pond nine-spined sticklebacks do not encounter piscine predators in their natural habitat, these plastic behavioural phenotypes have remained unexpressed for thousands of years, while the ability to perform the response has not been lost.

An earlier study found evidence of unexpressed behavioural phenotypes regarding the diversionary display in response to cannibalistic groups in three spined-sticklebacks (Foster *et al.*, 2019). The purpose of this conspicuous display is to draw cannibalistic groups away from the nest the male is guarding (Foster, 1994). Males from populations that do not encounter cannibalistic groups in nature had retained the ability to perform the diversionary display in some cases, even though the probability of doing so was lowered (Foster *et al.*, 2019). This raises interesting questions about the evolution of behavioural plasticity – it seems that potential for behavioural plasticity can be retained even when a particular phenotype is not adaptive in a new environment. Two explanations for this can be identified: either retaining the potential for unexpressed behavioural phenotypes is not costly and therefore not weeded out by natural selection, or the underlying genetic variation for behavioural plasticity is limited (due to genetic drift or otherwise). It is notable that in the study by Foster *et al.* (2019), the diversionary display was inhibited (albeit not lost) in populations without cannibalistic groups, whereas I found no evidence for inhibited response to predator in pond sticklebacks. A tempting explanation would be that the high level of drift caused by a small long-term effective population size in pond nine-spined stickleback populations making the evolution of inhibition

of ancestral expression less likely than in three-spined sticklebacks. However, the behavioural traits considered in these two studies are not necessarily comparable, and this study did not aim to directly quantify inhibition of expression, so conclusions should be drawn with caution.

It is interesting, that an activational plastic response to predation was found only in risk-taking during foraging and not in exploration, even though intuitively these traits could be thought to be related. Exploration could, therefore, be considered a less plastic personality trait than risk-taking. It could also be that inhibited exploration in the presence of predators is not adaptive in the wild. Exploration has often have been found to be correlated with aggressiveness in predator-sympatric populations (e.g. Dingemanse *et al.*, 2007) and predation is known to strengthen this correlation (Bell and Sih, 2007; Adriaenssens and Johnsson, 2013). It could be that variation in exploration reflects different antipredator strategies of equal fitness; in predator-sympatric environment, individuals could adopt either an "active" antipredator strategy, where they actively inspect their environment and opt for an escape response or aggression instead of hiding, or a "passive" strategy, where they do not attempt to actively gain information about their environment and are shy and cautious towards predators, staying in hiding. Since activity in an open field test has been linked to better survival in juvenile brown trout (Adriaenssens and Johnsson, 2013), the putative connection between exploration and overall activity might explain why some individuals retain high levels of exploration also in the presence of predators. Alternatively, it is also possible that exploration is more closely related to behaviour toward conspecifics (i.e. active investigation of and aggressiveness towards potential competitors) rather than antipredator behaviour, so that predators do not elicit a response in this behaviour.

Behavioural syndromes

Some populations expressed phenotypic behavioural correlations, but these did not fall into any discernible adaptive pattern, and genetic correlations were non-significant in all populations. Therefore, the hypothesis of adaptive behavioural syndromes was not supported. Adaptive behavioural syndromes related to predation have been demonstrated in three-spined sticklebacks (Bell, 2005; Bell and Sih, 2007; Dingemanse *et al.*, 2007), but similar results were not obtained in a study that investigated the behaviour of nine-spined sticklebacks (Herczeg, Gonda and Merilä, 2009). It may be, therefore, that the lack of behavioural correlations in nine-

spined sticklebacks is not merely due to the lack of statistical power, but due to these two species being different in the genetic architecture of their behaviour.

This study investigated two categories of behaviour; exploration that was not connected to feeding, and risk-taking during foraging, which involved feeding. In other studies, measures of exploration or boldness that do not involve food have frequently been found to be unrelated to measures that involve food. In the study by Herczeg *et al.* (2009) the latency to feeding was not correlated with exploration, and Dingemanse *et al.* (2007) found that exploration of novel foods was not part of the behavioural syndrome in contrast to exploration of novel environment. In rainbow trout, boldness measures that are related to foraging have been found to be correlated with each other, but not with a measure that was not related to foraging (Wilson and Stevens, 2005). In another study on the same species, exploration of a novel food and exploration of a novel non-food object were not correlated (Frost *et al.*, 2007).

Stamps (2007) has suggested that correlations arise between traits that have similar potential effects on growth and mortality, and these behavioural patterns are influenced by individual differences in growth rates. As exploration without a foraging context has a less straightforward relationship to growth and mortality than risk-taking during foraging, these behaviours do not necessarily contribute to the trade-off in a similar way and thus will not correlate with one another. This growth-mortality perspective on behavioural variation argues that individuals that have a high "preferred" growth rate will behave in ways that maximise their growth and thus take more risks to obtain food (Stamps, 2007). Indeed, the tendency to take risks during foraging has been found to be positively correlated with growth in three-spined sticklebacks (Ward *et al.*, 2004). Nine-spined sticklebacks are known to vary in their growth rates when food is abundant, with pond sticklebacks having higher growth rates than marine conspecifics (Välimäki and Herczeg, 2012). Since pond sticklebacks have decreased predation induced mortality, taking a lot of risks to forage should be strongly favoured in these populations, which appears to be indeed reflected in my results (see fig. 5).

The differences in risk-taking during foraging could thus reflect the differences in innate growth rates, whereas the differences in exploratory behaviour may reflect different antipredator strategies. As exploratory behaviour is often found to form a syndrome with aggressive behaviour (Bell and Sih, 2007; Dingemanse *et al.*, 2007; Adriaenssens and Johnsson, 2013), which I did not measure, differences in exploration might reflect different

antipredatory strategies – investigative and aggressive predator inspectors and cautious and peaceful hiders. In this study, even though high exploration was more common in pond populations, about half of the marine individuals were also explorative (see fig. 1a). This suggests that in a predator-sympatric environment, both explorative and cautious strategies exist and may have a comparable fitness effect in terms of survival.

Behavioural genetics of small populations

Both exploration and risk-taking were heritable in all populations. Heritability did not correlate with effective population size, suggesting that even small populations of nine-spined sticklebacks house enough behavioural genetic variation for these traits to evolve. Similarly, behavioural traits have been found to be heritable in both a large predator sympatric and a smaller predator naïve population of three-spined sticklebacks (Dingemanse *et al.*, 2009, 2012). The fact that the population differences in behavioural traits that were measured in common garden conditions indicate that this differentiation is genetically determined. However, a Q_{ST} – F_{ST} comparison would be needed to confirm whether the behavioural differentiation of pond populations has arisen as a result of natural selection as opposed to genetic drift alone.

Quantitative genetic variation did not correlate with estimates of effective population size, even though a non-significant trend towards this was observed. If the lack of a statistically significant result was due to insufficient statistical power, the observed pattern could be seen as being in line with the theoretical expectation of a relationship between effective population size and genetic variation. On the other hand, however, the lack of a statistically significant relationship implies interestingly enough that isolated pond populations of nine-spined sticklebacks have managed to retain behavioural genetic diversity that is comparable to the diversity found in marine populations in spite of very small population sizes. Similar results have been found regarding the genetic variance of morphological traits in nine-spined sticklebacks (Shimada *et al.*, 2011). Conversely, reduced additive genetic variance has been reported in a small predator naïve population of three-spined sticklebacks compared to a larger predator sympatric population (Dingemanse *et al.*, 2009). A compelling question is what kind of mechanism maintains variation in isolated pond populations of the nine-spined stickleback. In any case, these results further underline that the behavioural differences found in pond populations have been able to evolve as adaptations to relaxed predation pressure.

Local adaptation in nine-spined sticklebacks

If populations are locally adapted to specific environments, individuals should perform better in their native environment (Kawecki and Ebert, 2004). The most robust evidence can be obtained through reciprocal transplant studies that may not in some cases be methodologically feasible or ethically sustainable. This may especially be true for cases of local adaptation where different predation regimes play an important role. In this study, both pond and marine nine-spined sticklebacks were observed to reduce their risk-taking in the presence of predators. Additionally, marine sticklebacks, which have predators in their natural environment, took less risks overall than pond sticklebacks, which have evolved under a relaxed predator pressure for thousands of generations. Thus, we can see that the behavioural trend caused by acute predation risk was directionally the same as that caused by evolutionary history of predation risk, implying that the behavioural differentiation in risk-taking between marine and pond populations is due to predation.

In conclusion, this study has demonstrated that risk-taking behaviour in Fennoscandian nine-spined sticklebacks is genetically based, heritable, and shaped by environment – namely predation risk. I also demonstrated genetically based and heritable differences in explorative tendency between marine and pond populations, but predators did not have a plastic effect on this trait, thus the role of predation in shaping the differences in this trait is not clear. In any case, these results provide strong evidence of local behavioural adaptation in nine-spined sticklebacks. The results infer that genetically determined behaviour has evolved through natural selection, and that behavioural traits are well suited to studying local adaptation in general.

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SUPPLEMENTARY MATERIAL

Supplementary methods: Exploratory factor analysis of behavioural variables

We investigated the correlational structure of the behavioural variables by performing an exploratory factor analysis with varimax rotation for two factors across all populations using the *psych* package in R (ver. 1.9.12.31; Revelle, 2019). The Tucker Lewis Index of factoring reliability was 0.991 and the root mean square error of approximation (RMSEA index) was 0.061, indicating a moderately acceptable model fit. The two factors explained 81 % of variance in the data.

The first factor had a sum of squared loadings (SS loading) of 1.95 and explained 39% of the variance in the data. This factor described a gradient from cautious to explorative fish and had high positive loadings on the measures of time to head out and body out, low negative loadings on time spent in open area and number of feedings, and a low positive loading on latency to first feeding. The second factor had an SS loading of 2.08 and explained 42% of the variance in the data. This factor described a gradient from reluctant to eager feeders and had high positive loadings on time spent in the open area and number of feedings, high negative loading on latency to first feeding and low negative loadings on time to head and body out.

The factor structure was similar in all subpopulations, as well in both of the treatments. The strongest loadings on each of the two factors correspond to the two behavioural categories that we measured. All this suggests that the variables chosen to be analyzed correspond to two different categories of behaviour.

Supplementary figures

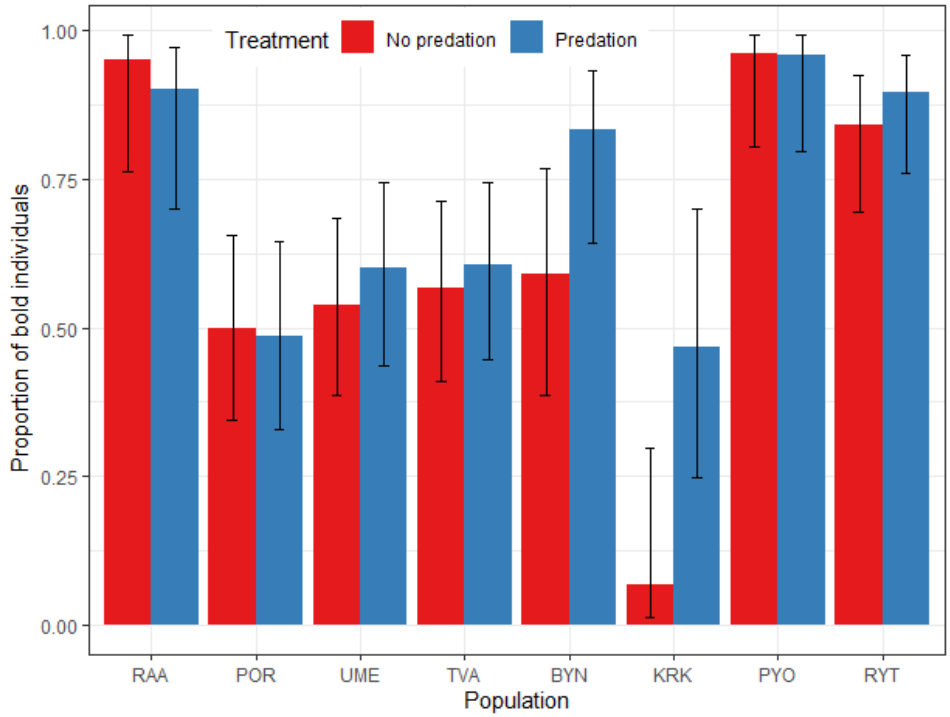

Fig. S1. Proportion of explorative individuals in different populations included in this study. For population abbreviations, see table 1. Vertical bars depict 95 % confidence intervals.

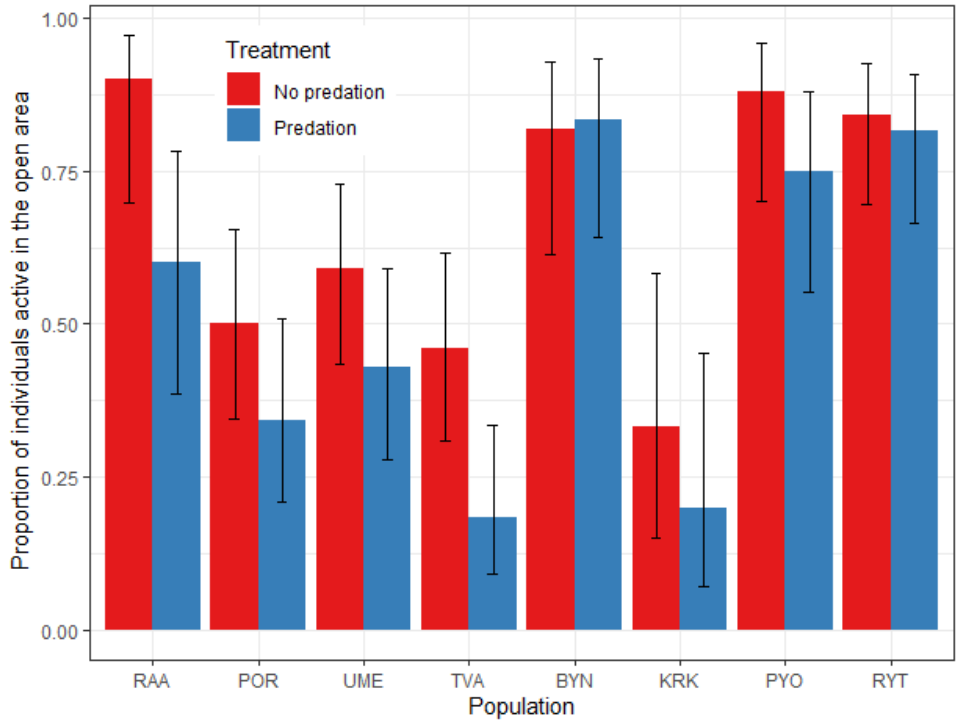

Fig. S2. Proportion of individuals spending time in the open area in each of the study populations. For population abbreviations, see table 1. Vertical bars depict 95 % confidence intervals.

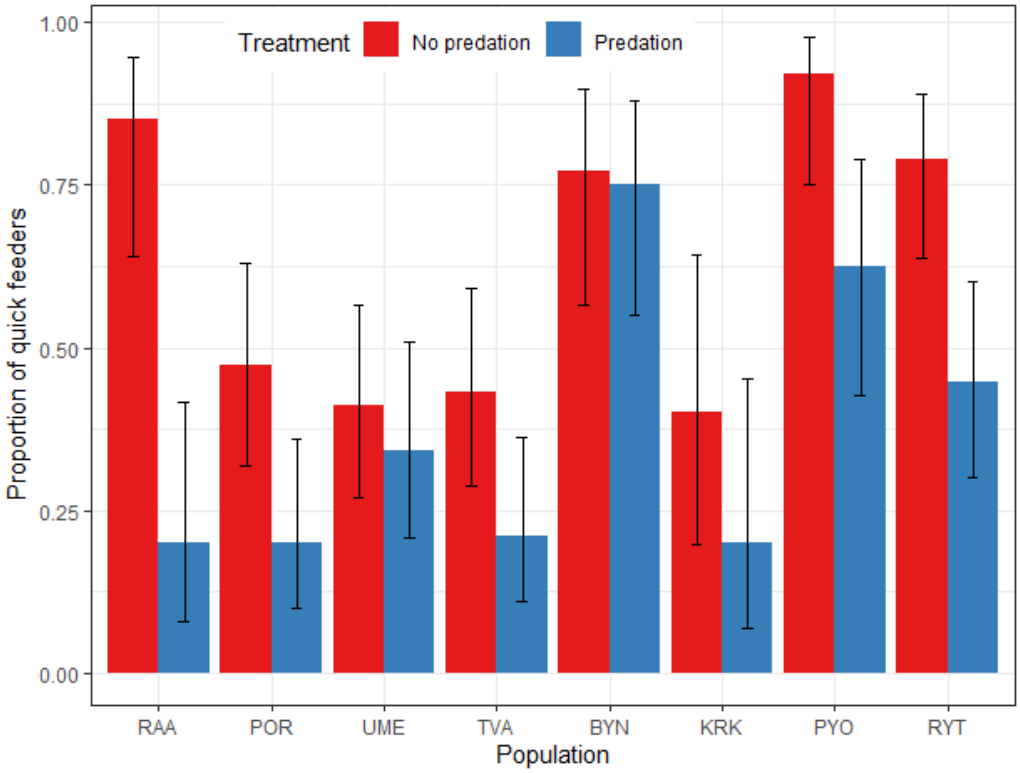

Fig. S3. Proportion of quickly feeding individuals in different populations. For population abbreviations, see table 1. Vertical bars depict 95 % confidence intervals.

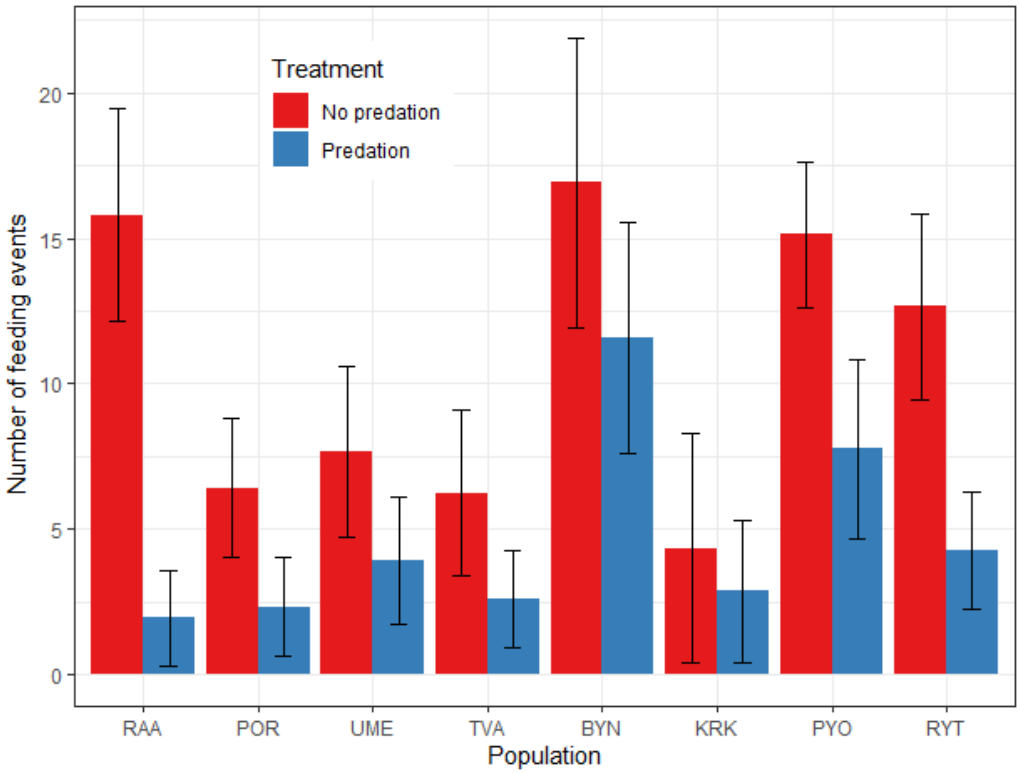

Fig. S4. Mean number of feeding events and 95% confidence intervals in different populations.

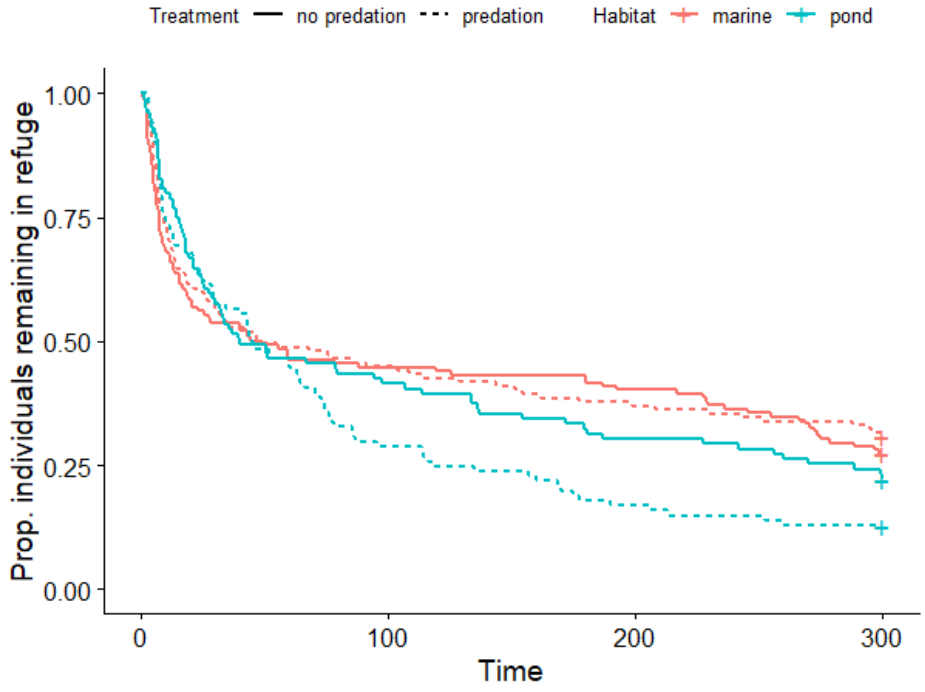

Fig. S5. Proportion of assessed individuals remaining inside the refuge at each time point (seconds) throughout the exploration trials in each habitat-treatment group, with"RAA" and "KRK" -populations included in the analysis.

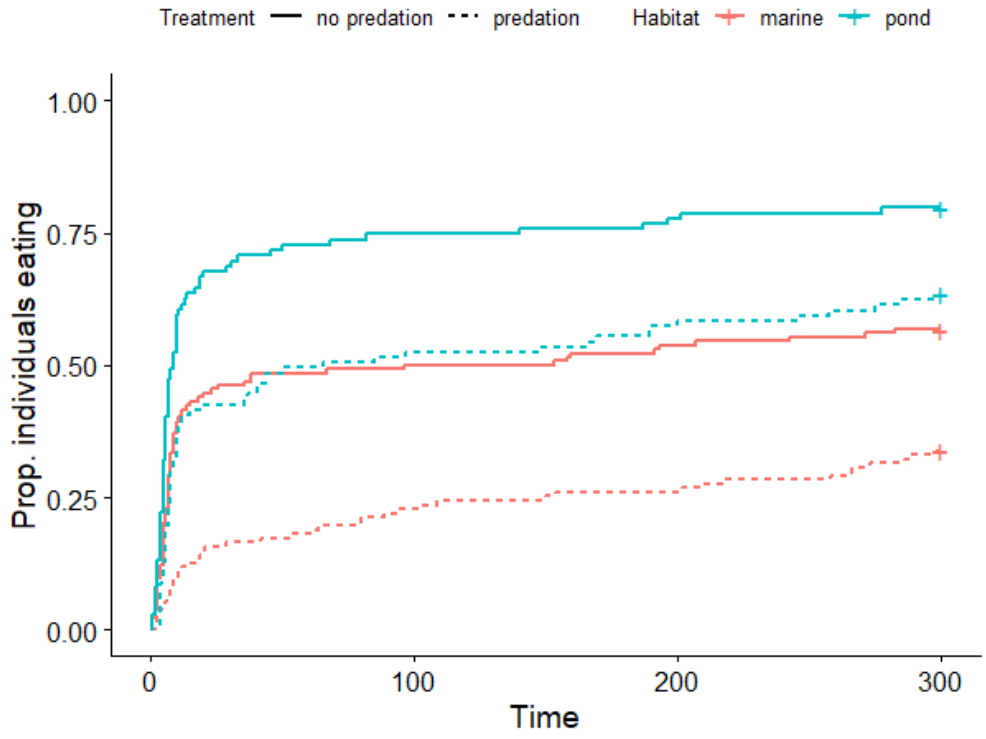

Fig. S6. Proportion of assessed individuals that had started eating at each time point (seconds) throughout the foraging trials in each habitat-treatment group, with"RAA" and "KRK" -populations included in the analysis.